REVIEW ARTICLE

Nuclear reprogramming of sperm and somatic nuclei in eggs and oocytes

Marta Teperek · Kei Miyamoto

Abstract Eggs and oocytes have a prominent ability to reprogram sperm nuclei for ensuring embryonic development. The reprogramming activity that eggs/oocytes intrinsically have towards sperm is utilised to reprogram somatic nuclei injected into eggs/oocytes in nuclear transfer (NT) embryos. NT embryos of various species can give rise to cloned animals, demonstrating that eggs/oocytes can confer totipotency even to somatic nuclei. However, many studies indicate that reprogramming of somatic nuclei is not as efficient as that of sperm nuclei. In this review, we explain how and why sperm and somatic nuclei are differentially reprogrammed in eggs/oocytes. Recent studies have shown that sperm chromatin is epigenetically modified to be adequate for early embryonic development, while somatic nuclei do not have such modifications. Moreover, epigenetic memories encoded in sperm chromatin are transgenerationally inherited, implying unique roles of sperm. We also discuss whether somatic nuclei can be artificially modified to acquire sperm-like chromatin states in order to increase the efficiency of nuclear reprogramming.

Keywords Epigenetic modification · Fertilisation · Nuclear reprogramming · Nuclear transfer · Spermatogenesis

M. Teperek · K. Miyamoto (✉)
The Wellcome Trust/Cancer Research UK Gurdon Institute,
The Henry Wellcome Building of Cancer and Developmental
Biology, University of Cambridge, Tennis Court Road,
CB2 1QN Cambridge, United Kingdom
e-mail: k.miyamoto@gurdon.cam.ac.uk

M. Teperek · K. Miyamoto
Department of Zoology, University of Cambridge,
Cambridge, United Kingdom

Introduction

During the course of development, totipotent embryonic cells differentiate and become committed to distinct lineages. Once a cell is differentiated, it cannot be dedifferentiated in normal development. However, it was proved that it is possible to dedifferentiate a cell experimentally in a process called cloning. Successful cloning experiments have been first performed in *Xenopus laevis*: when cells from embryos/tadpoles were transferred into enucleated eggs, arrested at the metaphase of the second meiotic division (MII; the term "MII egg" is used in frogs, while "MII oocyte" is used for mammals), healthy embryos with the genetic material of the donor cells were generated, reaching adulthood and sexual maturity [1]. Decades later, Dolly the sheep was born as the first mammalian somatic cell nuclear transfer (SCNT) animal [2], followed by reports of successfully cloned individuals across many mammalian species, including the mouse [3], cow [4], goat [5], pig [6, 7], cat [8], rabbit [9], horse [10], rat [11], dog [12], ferret [13], red deer [14], and camel [15]. However, regardless of the species used, embryos obtained by SCNT develop to the adulthood inefficiently compared to embryos obtained by fertilisation. Furthermore, it is generally regarded that the more differentiated somatic cell nuclei are when used as donors in SCNT, the less efficient the process is; for example, in frogs, when blastula or gastrula nuclei were used as donors for SCNT, 36 % of resulting embryos were able to develop to feeding tadpoles, whereas when the nuclei of intestinal epithelium were used, only 1.5 % embryos reached a feeding tadpole stage [16]. Although this rule may not be fully applied to mammalian cloning [17, 18], it is still valid to conclude that embryonic cells are a better source for SCNT [19].

Why is SCNT inefficient or, in other words, why are somatic cells resistant to reprogramming? The sperm which fertilises the egg is also a highly specialised cell. However, despite being specialised, its nucleus can almost invariably support normal embryonic development. Therefore, the specialisation of the sperm that occurs during spermatogenesis prepares it to undergo efficient reprogramming by the egg after fertilisation. Namely, the sperm is developmentally 'programmed' to be 'reprogrammed' and to sustain normal embryonic development. In this review we first describe sperm 'programming': we review the important nuclear changes occurring during spermatogenesis that allow the mature sperm to acquire its unique chromatin features. Secondly, we discuss which of the unique sperm features may be responsible for the high efficiency of embryonic development after fertilisation, with a focus on protein and RNA contents, as well as on epigenetic modifications present on the chromatin of the mature sperm. Thirdly, we describe reprogramming events occurring upon NT in order to compare them to those occurring at fertilization. Thereafter, we summarise the most commonly observed abnormalities in cloned embryos, which are largely attributed to features of somatic cell chromatin. Lastly, we focus on the main differences between sperm and somatic cells that are likely related to reprogramming efficiency and discuss possible ways of improving cloning efficiency by making somatic cells more sperm-like.

Unique features of sperm chromatin

Sperm specialisation occurs during spermatogenesis as a series of precisely controlled events changing a progenitor germ cell into a spermatozoon. The length of the process, as well as the precise mechanisms controlling each step of spermatogenesis, differs amongst vertebrates, though general concepts are similar [20]. Somatic precursors of sperm cells, spermatogonia (developed from male germ cells), can undergo either a proliferative or a differentiative cell division. The former produces more spermatogonial cells, the latter results in the formation of a spermatogonial cell and a primary spermatocyte [21]. Primary spermatocytes enter meiotic division, producing secondary spermatocytes, which, upon completion of the meiotic division, form spermatids [20]. Spermatids have the number of chromosomes and the DNA content already reduced and do not undergo any other divisions. However, they resemble somatic cells morphologically, as well as at the molecular level, and in order to transform into highly specialised mature sperm they have to complete a series of substantial structural and morphological changes, called spermiogenesis [22] (Fig. 1).

Changes in the nuclear composition

One of the most striking changes occurring during spermiogenesis is the compaction of the sperm nucleus. Interestingly, it has been calculated that the volume of DNA of mouse sperm is six times smaller than the DNA in mitotic chromosomes [23]. The high condensation of sperm DNA is possibly due to the presence of protamines. Protamines are small and highly basic proteins that become incorporated into the chromatin during spermiogenesis in place of core histones that are the major component of the chromatin in spermatids [24]. The process of protamine incorporation is complex and requires many intermediate steps. Firstly, histone variants are thought to be incorporated alongside canonical histones, which are subsequently modified post-translationally and replaced by transition proteins (explained later) [25, 26]. Eventually, transition proteins are replaced by protamines, which are the major chromatin component of the mature sperm (Fig. 1). Furthermore, there are a lot of other changes occurring: transcription ceases, and a lot of proteins disappear from the maturing sperm nucleus whereas others are specifically accumulated. Below, we briefly describe our current understanding of nuclear changes occurring during spermiogenesis.

Histone variants and histone modifications

As mentioned above, incorporation of histone variants is the first major event allowing the nuclear maturation of a spermatid. There are numerous histone variants expressed specifically in testis, for example H2A.lap and H2A.Bbd as variants of histone H2A [27, 28], TH2B as a variant of H2B [29, 30], H1t and Hils1 as variants of H1 [31, 32] or H3t as a variant of histone H3 [33]. It has been shown that H2A.lap1 marks transcriptional start sites of specific transcripts expressed during spermatogenesis [28]. Interestingly, other histone variants are thought to facilitate incorporation of protamines. In vitro studies, in which nucleosomes have been assembled with testis-specific histone variants (H3t, TH2B, H2A.L2) and with somatic-type histones, indicate that such combinatorial nucleosomes are unstable [34–36]. Therefore, it is hypothesized that when these histones start to be expressed during spermatogenesis they form nucleosomes with somatic-like histones present in the spermatids. This, in turn, confers the instability of such combinatorial nucleosomes and allows the incorporation of transition proteins [37]. Furthermore, the instability of histone-containing nucleosomes can also be due to the presence of post-translational modifications that can alter the chromatin-binding properties. Such modifications can be present not only on core histones, but also on testis-specific histone variants [38–40]. One of the

Fig. 1 Major chromatin changes during spermiogenesis. In order to achieve a sperm-like chromatin state, the round spermatid which enters the spermiogenesis process undergoes a series of chromatin remodelling events. First, canonical core histones packing the chromatin in the round spermatid can be replaced by histone variants, which together with global histone acetylation, leads to instability of the nucleosome structure. Subsequently, transition proteins are incorporated in place of unstable nucleosomes in the elongating spermatid. Finally, transition proteins are replaced with protamines. The mature sperm chromatin is mainly composed of protamines, with interspersed histones and with tightly associated mRNAs and transcription factors. All these processes are occurring in parallel with the cessation of transcription—round spermatids are transcriptionally active, whereas no transcription is detected in the mature sperm
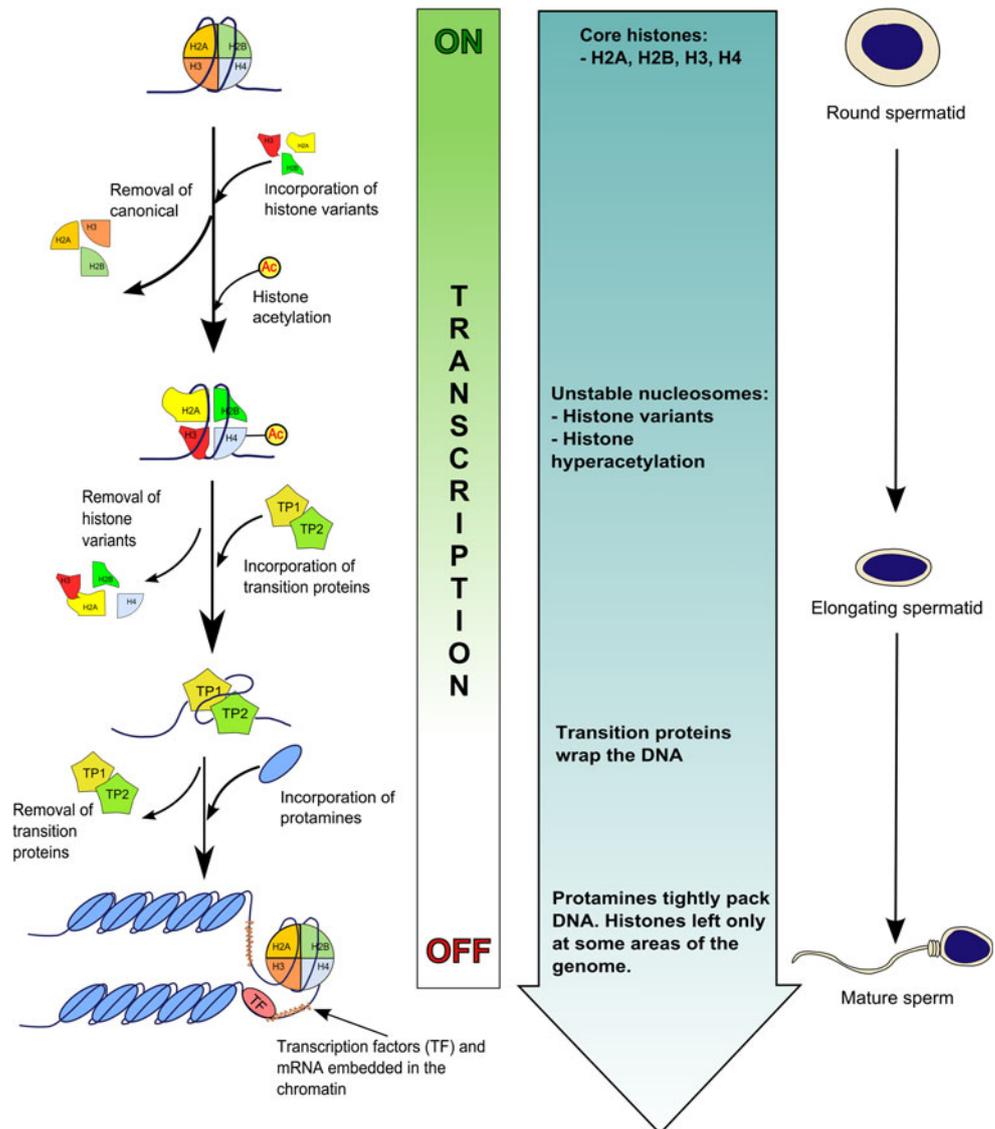
most striking and the best described changes in histone post-translational modifications is a wave of core histone acetylation during spermiogenesis. Immunohistochemical studies revealed that acetylated forms of H2A, H2B, H3 and H4 are present specifically in elongating spermatids (no acetylation in round spermatids and no acetylation in mature sperm), suggesting that the transient presence of this mark may be implicated in exchange of histones to protamines and condensation of sperm DNA (Fig. 1) [41]. Further studies led to the identification of a protein, named Brdt (Bromodomain testis-specific), which specifically binds to acetylated lysine residues on histone H4 [42, 43]. Studies using mammalian culture cells ectopically expressing Brdt have shown that its binding to acetylated histones leads to chromatin compaction [42]. It has been also demonstrated that Brdt remodels chromatin in an ATP-independent way and that it interacts with Smarce1,

which is a member of SWI/SNF remodelling complex [44]. Importantly, male mice lacking Brdt are infertile and have abnormal, misshapen sperm [45], suggesting that Brdt binding to acetylated histones may be indeed a crucial step allowing a proper condensation of sperm chromatin.

Transition proteins and protamines

Apart from histones and their modifications, other factors which are implicated in protamine deposition are transition proteins. Global histone acetylation precedes their appearance [37]. There are two transition proteins: transition protein 1 (TP1) and transition protein 2 (TP2). Their function is redundant, since mice with deletion of TP1 or TP2 alone are fertile (albeit with a reduced fertility) [46, 47]. However, knockout of both TP1 and TP2 results in infertile mice with abnormal sperm [26], suggesting that

transition proteins are important for a proper spermatogenesis. Finally, the protamines, which replace transition proteins towards the end of spermiogenesis, are also essential for male fertility. It has been demonstrated that even heterozygous male mice, which are mutant for protamine 1 or protamine 2, are infertile [48]. Detailed analyses of protamine 2 mutant mice revealed that haploinsufficiency of protamine 2 affected the DNA integrity of the mature sperm [49]. Interestingly, abnormal levels or mutations of protamines also correlate with infertility in humans [50, 51].

Cessation of transcription and disappearance of basal transcriptional machinery

Spermiogenesis is however not only a process leading to the acquisition of unique sperm-specific proteins. Numerous proteins typical for somatic cells disappear in the course of spermiogenesis. The most remarkable is the disappearance of RNA polymerase II and hence, cessation of transcription in maturing spermatids; round spermatids are actively transcribing, while there is no transcription in mature sperm (Fig. 1) [52]. The same happens to basal transcription factors: TAF1 and TLF (TBP-like factor) also disappear during spermatid condensation [53–55]. Therefore, it could be that cessation of transcription and removal of the majority of basal transcriptional machinery is important for the paternal nucleus to sustain the embryonic gene expression pattern after fertilization.

Which components of the sperm make it 'programmed' to be 'reprogrammed'?

Since there are numerous factors being acquired and lost during sperm maturation (Fig. 1), it is not easy to dissect the ones that are responsible for rendering the sperm easily reprogrammable by eggs/oocyte, as opposed to the somatic cell after nuclear transfer. We can however classify them into five categories: (1) protamines, (2) sperm-derived transcriptional regulators, (3) sperm-derived RNAs, (4) post-translational modification of histones in the mature sperm, and (5) DNA methylation profiles in the mature sperm. We will discuss below the potential involvement of each of these categories in making sperm easily reprogrammable.

Protamines

One of the biggest differences between sperm and somatic cells is the fact that DNA of a somatic cell is wrapped around histones, whereas DNA of sperm is tightly packed by protamines. Containing protamines instead of canonical

histones can have at least two roles in facilitating the reprogramming at fertilisation. Firstly, after fertilisation, the protamines from the sperm are removed by an egg/oocyte with the aid of a maternal protein called nucleoplasmin [56–59]. Subsequently, oocyte-derived histones are incorporated into paternal DNA to allow the assembly of chromatin [60, 61]. On the other hand, a somatic cell does not have protamines, and hence it may be more difficult for the transplanted somatic cell to exchange its histones for the oocyte-derived ones. In addition, somatic cell histones often bear post-translational modifications associated with active gene states, according to the lineage from which the cell is originated. As a result, some genes characteristic for that lineage could continue to be inappropriately expressed in the embryo. Indeed, it has been shown in *Xenopus* that upon NT of somatic cells derived from somites (muscle precursors expressing a gene called *MyoD*), resulting embryos continued to aberrantly express *MyoD* (discussed in the later section) [62]. Therefore, it is tempting to speculate that having protamines instead of histones may be beneficial for sperm to erase the developmental program which is often encoded in histone marks: this is because at fertilisation protamines are efficiently replaced with oocyte-derived histones and because the newly incorporated histones are likely to be modified according to the embryonic developmental program. Indeed, it has been shown that embryos derived from round spermatid injection (round spermatids do not yet have protamines deposited on their chromatin) display epigenetic abnormalities as compared to embryos obtained by mature sperm injection (which do have protamines on their DNA): round spermatid-derived embryos have been shown to have elevated DNA methylation levels and histone H3K9me3 marks present on the paternal chromatin, which are not normally observed in sperm-derived embryos [63]. Apart from these potential roles of protamines in efficient histone exchange, the presence of protamines on sperm DNA and its tight packaging likely protects the DNA from any physical damage. It has been shown that in rabbits in which no offspring could be derived after the protamine-free round spermatid injection, the developmental arrest is likely due to abnormal ploidy of the resulting embryos [64]. Similarly, during the NT procedure somatic cells are exposed to numerous micromanipulations, and because they do not have protamines tightly protecting their DNA, these procedures could lead to DNA damage. In fact, it has been suggested that one of the major causes of developmental arrest of NT embryos is a result of DNA loss [65], which might be a consequence of DNA damage. With the current state of knowledge it is therefore difficult to discriminate whether the presence of protamines on the DNA helps to epigenetically program the paternal chromatin for embryonic development or whether it prevents the DNA

from physical damage (or both). In addition, it has been shown that even though the embryonic development after round spermatid injection is generally less efficient than the development after sperm injection, it can reproducibly give rise to normal offspring in several species [66–70], suggesting that the chromatin of round spermatid, even though it is not protaminated, can support full term development. However, since mice deficient with protamines are infertile [48], having protamines on the DNA is clearly beneficial for the development and hence the lack of protamines might be one of the explanations for the low efficiency of SCNT.

Sperm-derived transcriptional regulators

As mentioned before, it has been demonstrated that towards the end of spermiogenesis, transcription ceases and components of the basal transcriptional machinery disappear [55]. However, it has also been shown that the mature sperm contains transcription factors (Oct-1, Ets-1, C/EBP and TBP) associated with the hypersensitive regions of chromatin [71] (Fig. 1). It is therefore likely that such chromatin-associated factors could be delivered to the oocyte at fertilization. Furthermore, proteomic analysis of the mature sperm led to the identification of more proteins which are involved in transcriptional regulation, for example, Bromodomain-containing protein 7 (Brd7) or Polycomb protein Suz12 [72]. Upon delivery to the oocyte, these factors might help to pattern gene expression characteristic for the developing embryo. In contrast, a somatic cell during a nuclear transfer procedure may deliver transcriptional regulators responsible for the maintenance of its own differentiated cell state to the oocyte. This might interfere with normal embryonic gene expression since the embryo requires factors enabling the establishment of totipotency and not cell-type specific, differentiation factors. In fact, injection of the somatic cell cytoplasm into oocytes impairs normal development of fertilised embryos and indeed it has been demonstrated that such embryos have decreased expression levels of pluripotency factor Oct4 [73].

Sperm-derived RNAs

It has been recently discovered that mature sperm, despite having a very low amount of cytoplasm, carries numerous mRNAs [74], including mRNAs encoding transcription factors, for example cMyc [75]. These mRNAs have been shown to be delivered to the oocyte after fertilisation [76]. What is the role of these sperm-derived mRNAs? It is currently not well understood, but there are several hypotheses. The first one suggests that these mRNAs remain tightly associated with sperm chromatin and can

somehow protect some regions of the sperm DNA from protamination. As a result, this would allow histone retention on these regions. Such protamine-free regions could be then selectively activated in the developing embryo (see section below) [74]. A second hypothesis is that RNA stored in the sperm can exert influence on the gene expression profile of the resulting embryo. It could be either by providing mRNA which serves as a template for synthesis of transcription factors important for embryogenesis, such as cMyc, or by inducing paramutations. Paramutation is a heritable epigenetic change resulting in a mutant phenotype in the absence of the actual genetic mutation. For example, it has been shown that wild type progeny of mice generated from heterozygous fathers harbouring a mutation in a *cKit* gene (demonstrated with a white tail phenotype) also have white tails. This paramutation was shown to be induced by antisense RNA carried over to the oocyte by the sperm at fertilisation [77]. It is also possible that sperm-derived RNA can be directly involved in regulating embryonic development. Indeed, it has been recently shown that sperm-borne microRNA-34c is required for the first cleavage division of the mouse embryo by directly regulating antiproliferative protein Bcl2 [78]. Therefore, there is evidence that carry-over sperm RNA can influence the pattern of embryonic gene expression and even phenotypes of the progeny. Such instructive information about embryonic development in a form of RNAs is likely to be absent or altered in somatic cells (presence of somatic-cell specific RNAs), therefore affecting the normality of embryonic development after NT.

Post-translational modifications of histones in the mature sperm

Another explanation for why the sperm so efficiently supports embryonic development can be due to the presence of particular post-translational modifications on histones retained in mature sperm. As explained before, towards the end of spermiogenesis, histones are replaced with protamines. However, it has been recently shown that both in mice and in humans some histones remain in the mature sperm [79, 80]. It has also been described that both in mouse and in human, sperm-derived histones (at least some of them) are transmitted to the oocyte at fertilization and retained in the paternal pronucleus [61, 81, 82]. Moreover, retention of histones in the mature sperm is nonrandom; namely, histones are retained at promoter regions of developmentally important genes. Furthermore, these retained histones bear epigenetic marks and their patterns correlate well with future embryonic gene expression: genes with an activating mark, H3K4me3, are switched on early in development, whereas genes with a

repressive mark, H3K27me3, are either repressed or activated late [79, 80]. Interestingly, this phenomenon has also been observed in species in which sperm is not protaminated (core histones are the major component of chromatin in the mature gamete). For example, in zebrafish, which does not have protamines, developmentally important promoters also have signatures of H3K4me3 and H3K27me3, which correlate with future embryonic gene expression [83, 84]. Interestingly, it has been described that infertile patients have abnormal histone retention profiles [85]. All these suggest that sperm may be able to provide the embryo with a 'patterning' message, encoded in its histone marks, as to which genes should be expressed and when during embryogenesis. Sperm of infertile patients, which do not have this message (or have incorrect messages), cannot support proper embryonic development. Such lack of the correct patterning message could also explain inefficient embryonic development after nuclear transfer. Somatic cells have their epigenetic marks on histones established according to their own developmental profile, and those epigenetic marks are likely different from those of embryos.

DNA methylation profiles in the mature sperm

Another epigenetic feature of the mature sperm that may render it suitable to support embryonic development is DNA methylation. Cytosine in DNA can be methylated, which leads to creation of 5-methylcytosine. Methylated cytosines are often associated with gene repression [86–90], whereas unmethylated cytosines usually mark transcriptionally active genes [91, 92]. Differential cytosine methylation is also observed at gene promoters in mature sperm. Interestingly, cytosine methylation states in gene promoters in sperm are very similar to those of embryonic stem cells [93] and correlate with gene expression patterns in the embryos. Genes whose promoters are unmethylated in the mature sperm are expressed early during embryogenesis, whereas methylated genes are expressed late or repressed in embryogenesis [79, 80, 84]. Moreover, there is a correlation between abnormal DNA methylation patterns in the mature sperm and infertility in humans [94]. Therefore, DNA methylation can be yet another way for the sperm to contribute a developmental message to the embryo, another way the sperm differs from somatic cells, and which may explain the low efficiency of SCNT. In fact, a recent genome-wide study clearly indicates that DNA methylation patterns of SCNT embryos resemble those of donor somatic genome rather than those of the paternal genome of fertilised embryos [95], suggesting that DNA methylation profiles are indeed different between sperm-derived and somatic cell-derived embryos.

Reprogramming of somatic nuclei in eggs and oocytes

We have so far discussed how sperm nuclei are efficiently reprogrammed in eggs/oocytes. However, cloned embryos are produced in a very different way from normal fertilised embryos and also undergo unique reprogramming processes. In this section we describe a series of reprogramming events in cloned embryos. To produce a cloned embryo, nuclear transplantation of a somatic nucleus to an enucleated egg/oocyte is performed (Fig. 2). Eggs/oocytes at the metaphase II (MII) stage are widely used as recipients for NT. Since the MII-arrested oocytes have a strong maturation promoting factor (MPF) activity, the transplanted somatic nucleus undergoes nuclear envelope breakdown and premature chromosome condensation (PCC) (Fig. 2) [96, 97]. It seems that exposure of somatic chromatin to the MII egg/oocyte cytoplasm is an important determinant for successful reprogramming by increasing the number of origins of DNA replication in order to facilitate robust DNA replication cycles in embryos [98, 99]. It has also been postulated that PCC allows oocyte reprogramming factors to gain access to somatic chromatin since the barrier of nuclear envelopes disappears. Epigenetic modifications on histone tails, such as deacetylation and phosphorylation [100, 101], are also induced soon after NT (within 2 h) (Fig. 2). Moreover, some core and linker histones are rapidly removed from somatic chromatin or exchanged to oocyte counterparts (Fig. 2). For example, macroH2A, associated with repressive chromatin, is readily removed after NT (Fig. 2) [102]. Subsequently, MII-arrested NT oocytes are activated and start early embryonic development. NT embryos then form pseudo-pronuclei, where somatic chromatin undergoes extensive decondensation (Fig. 2) [97]. In mouse, heterochromatin reorganization is induced in the pseudo-pronuclei of NT embryos [103]. Additionally, active DNA demethylation of the somatic genome is observed during the one-cell stage (Fig. 2) [95, 104]. Histone modifications of the pseudo-pronuclei are significantly different from those of original somatic chromatin and more resemble those of fertilised pronuclei, although quantitative differences of such histone modifications have been reported [100, 105–107] (see below). Towards the end of the first cell cycle in mouse, transcriptional activators are gradually accumulated in pronuclei (Fig. 2) [108] to allow major embryonic gene activation at the 2 cell stage (Fig. 2). Subsequently, genes required for pluripotency such as *Oct4* and *Nanog* are activated [109]. Once NT embryos have reached the blastocyst stage, embryonic stem cells can be derived. It is possible that reprogramming is continuing even at the blastocyst stage because abnormal histone modifications prominent at the early embryonic stages are corrected in the blastocyst stage embryos [107].
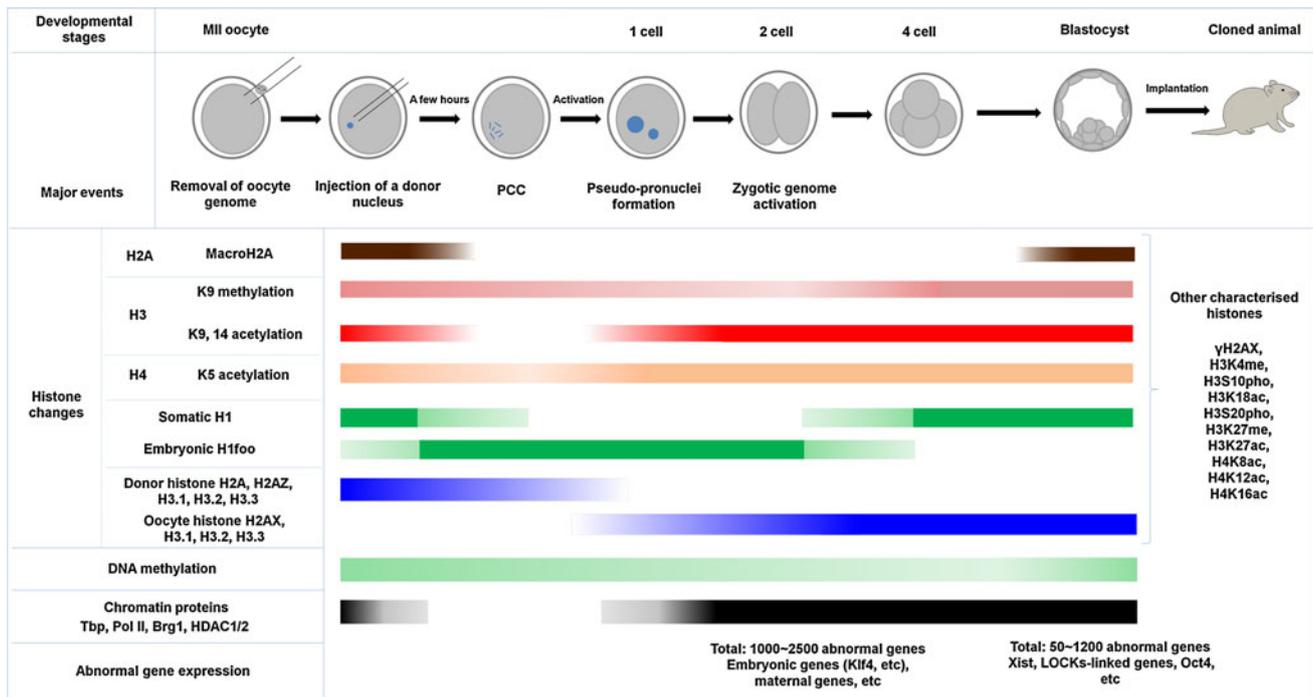
Fig. 2 Nuclear reprogramming in somatic cell nuclear transfer embryos. A series of reprogramming events occurring in NT embryos are depicted (mainly focusing on mouse NT embryos). A somatic nucleus injected into an enucleated oocyte at the metaphase II stage undergoes premature chromosome condensation (PCC). After activation, pseudo-pronuclei are formed. Transcription of embryonic genes starts from the 2-cell stage. Cloned embryos can be implanted to foster mothers and in most cases less than a few percentage of embryos can develop to term. Histone modifications, histone variants, DNA methylation, and chromatin proteins show dynamic changes during preimplantation development. Many of them are important for development of cloned embryos, but are abnormally regulated compared to fertilised counterparts. Abnormal gene expression in cloned embryos has also been reported. The numbers of abnormally expressed genes are based on transcriptome studies of 2-cell embryos [151, 152] and those for blastocyst embryos [150, 151, 156]

There are some alternative methods for NT. Oocytes at the telophase stage and enucleated embryos at the one-cell stage can also be used as recipients for NT as long as pronuclear factors are not removed during enucleation [110–112]. It is not clear whether reprogramming events induced in these NTs are the same as the ones in NT to metaphase II oocytes. Apart from these NTs, which can support full-term development, somatic nuclei injected into the giant nucleus of *Xenopus* oocytes at the meiotic prophase I stage also known as the germinal vesicle (GV) stage can be reprogrammed to express embryonic genes [113]. This type of NT is adequate for analysing mechanisms of transcriptional reprogramming [114, 115] since it does not require cell divisions and new protein synthesis. However, this type of NT does not support full term development.

There are a few maternal factors known to be important for development of NT embryos. For example, nucleoli in mouse and porcine NT embryos are derived from the nucleolus of the recipient oocyte [116]. When maternal nucleoli are removed, NT embryos are deprived of nucleoli and fail to develop. These results suggest that maternally inherited nucleoli are necessary for development. Tpt1 protein is an example of another maternal factor important for development of bovine NT embryos with the mechanisms still unknown [117]. Additionally, a screen for maternal factors required for reprogramming has identified DJ-1 as a maternal protein required for development of porcine NT embryos [118]. DJ-1 inhibits P53 activation in NT embryos; this also suggests that P53 is prone to be activated in NT embryos. Furthermore, it has been shown that maternal Tet3-mediated hydroxymethylation is induced in cloned mouse embryos [119] and is responsible for DNA demethylation on the Oct4 promoter [120]. More studies to identify maternal factors important for development of cloned embryos are needed for understanding the basis of egg/oocyte-mediated reprogramming.

Abnormalities in cloned embryos in relation to somatic cell characteristics

A number of reprogramming events, discussed in the previous section, are induced in NT embryos. NT embryos

that have successfully gone through these events can achieve full-term development. However, it is often the case that NT embryos fail to complete at least a part of reprogramming events. In this section, we discuss what kinds of errors are prone to occur in NT embryos during reprogramming. We then relate the defective reprogramming events in NT embryos to specific characteristics of somatic chromatin.

DNA demethylation and histone modification

In mouse, upon fertilisation, sperm DNA undergoes active and global demethylation during the one-cell stage. Low methylation levels are maintained until the blastocyst stage when global remethylation starts [121]. In cloned embryos, the extent of DNA demethylation in the transplanted somatic nuclei is less than that in paternal pronuclei [104]. Such abnormally high DNA methylation states in cloned embryos have been found across many mammalian species [104, 122, 123]. Bisulfite sequencing analyses have revealed that high DNA methylation states remain at the various genomic regions including the centric and pericentric repeats [124] and the *Oct4* regulatory regions [125, 126]. A recent genome-wide DNA methylation study has identified more than 20 genes whose DNA methylation remains abnormally high after NT compared to fertilised embryos [95]. This study also shows that some repetitive elements, such as long interspersed elements (LINEs) and long terminal repeats (LTRs), are resistant to DNA demethylation in cloned embryos [95]. Since these genomic regions are efficiently demethylated in paternal pronuclei, the refractory nature towards DNA demethylation seems largely attributed to somatic genomes.

Histone modifications of somatic cell chromatin are dramatically changed after NT to oocytes [100, 101]. Although histone tails of somatic chromatin are reprogrammed to resemble those of embryonic chromatin, a number of abnormal histone modifications have been reported in NT embryos [100, 105–107, 127]. Notably, abnormally high levels of histone H3K9 methylation are retained in NT embryos [100, 105, 107] and the high H3K9 methylation is likely one of the reasons why cloned embryos exhibit a low developmental capacity. Indeed, removal of H3K9 methylation prior to NT improves cloning efficiencies [128, 129] although causal relationship between loss of H3K9 methylation and reprogramming in NT embryos has to be further tested. H3K9 methylation also restricts reprogramming in iPS cells [130, 131], supporting the idea that somatic chromatin acquires H3K9 methylation to stabilize differentiation states and hence to resist nuclear reprogramming. A more targeted way of removing histone marks before NT,

such as locus-specific H3K9 methylation removal, may further boost reprogramming efficiency. It has also been shown that modulating histone acetylation levels by histone deacetylase inhibitors can greatly improve cloning efficiencies [17, 132–136]. For example, histone H4K5 in NT embryos shows less acetylation than in fertilised embryos [127] and this abnormal acetylation state can be partially corrected by Trichostatin A treatment [137]. Furthermore, histone deacetylase inhibitors also improve many aspects of reprogramming including transcription and chromatin reorganization [100, 138, 139]. Histone modifications on H3K27 play an important role in gene regulation especially in relation with polycomb complexes. Many somatic genes are repressed through H3K27 methylation. After NT of somatic cells to oocytes, higher H3K27 methylation [140] and lower H3K27 acetylation [106] than in fertilised embryos are observed in 1- and 2-cell stage embryos. In addition, localisation of PRC2 components in cloned embryos is significantly different from fertilised embryos [140] and abnormal expression of polycomb-associated genes is observed [141]. Trichostatin A treatment was shown to increase a H3K27 acetylation level in cloned embryos to the one of fertilised embryos [106]. It would be interesting to see whether the elevated level of H3K27 acetylation is linked to the improvement of other chromatin signatures, such as H3K27 methylation and association of polycomb complexes.

In addition to the above mentioned histone modifications, linker histones and histone variants show dynamic changes upon NT of somatic cells to oocytes. The somatic type linker histone H1 is rapidly exchanged with the embryonic type linker histone B4/H1foo upon NT to oocytes [142–144]. Incorporation of histone B4 into the somatic chromatin creates accessible states of chromatin [144, 145]. A recent study indicates that histone variants H3.1, H3.2 and H3.3, as well as H2A and H2A.Z, are rapidly removed from transplanted nuclei in mouse NT embryos [146]. At the same time, oocyte-derived histone H3 variants and H2AX are incorporated into the transplanted nuclei. Interestingly, abnormally high amounts of H3.1 are incorporated into transplanted somatic nuclei compared to fertilised embryos (Fig. 2) [146]. This might be attributed to the features of donor chromatin that prefer H3.1 incorporation for heterochromatin formation or by the fact that chaperones for H3.1 can be carried over with the donor cell. In accordance with the incorporation of oocyte-derived H2AX into transplanted nuclei [146], phosphorylated H2AX (γH2AX) is found in pseudo-pronuclei of NT embryos although the number of γH2AX foci is smaller than that of fertilised embryos [147]. γH2AX in 1-cell embryos is proposed to be associated with DNA repair and DNA demethylation.

Embryonic gene activation

Transcriptionally silent NT embryos start to express embryonic genes from the time of zygotic genome activation (ZGA) onwards. Hundreds of embryonic genes that are silenced in somatic cells can be activated in NT embryos. However, many defects in properly turning on embryonic genes have been reported [148–152]. Abnormal gene expression in NT embryos is observed as early as ZGA [151, 152]. Microarray analysis of 2-cell mouse embryos has revealed that more than 2000 mRNAs are misregulated in NT embryos compared to fertilised embryos [152]. Transcription by RNA polymerase I is also disturbed in cloned embryos around this stage [139, 153]. This early abnormal gene expression can be rescued by treatment with histone deacetylase inhibitors [135, 139], suggesting that abnormal gene expression in early embryos is at least partially due to the somatic epigenome, which is not adequate to support the embryonic gene expression program. In fact, some somatic genes continue to express in NT embryos at the 2 cell stage [154]. Equally importantly, maternally stored transcripts are not properly degraded in NT embryos [152]. This is also true in rhesus-bovine interspecies NT embryos [155], implying that inappropriate degradation of maternal transcripts can cause a critical problem for subsequent embryonic development. Although NT embryos tend to display numerous abnormalities at the early developmental stages, the number of abnormally expressed genes in cloned embryos usually decreases at the blastocyst stage [151, 156]. This could be partially because only successfully reprogrammed embryos are able to reach the blastocyst stage. Nevertheless, these results support the idea that reprogramming is continued throughout early embryonic development. However, there are some embryonic genes that are resistant to reprogramming even at the blastocyst stage, such as Oct4 [148] and Sox2 [150]. Abnormal expression of such important pluripotency genes at this stage may severely affect subsequent development after implantation.

Recently, large-scale transcriptome analyses have identified gene loci that are often abnormally regulated in cloned blastocyst embryos [150, 157]. Genes in the "large organized chromatin K9-modifications" (LOCKs) regions are repressed in NT embryos. LOCKs are enriched with histone H3K9me2, a repressive mark [158]. Differentiated cells exhibit larger occupancy of the LOCKs in the genome than embryonic cells [158]. Since H3K9 methylation is difficult to remove in cloned embryos (see above), it is plausible that H3K9 methylation in LOCKs of somatic chromatin causes abnormal gene repression in NT embryos. It would be interesting to test functional significance of the abnormal gene repression associated with LOCKs in NT embryos. Another set of abnormally regulated genes is X chromosome-linked genes. This is due to the ectopic expression of Xist from the active X chromosome [157]. Suppressing excess Xist RNA by gene knockout [157] or siRNA-mediated knockdown [159] greatly improves the cloning efficiency. These results indicate that misregulation of a single important gene can cause detrimental effects on development of cloned embryos.

Epigenetic memory of somatic chromatin in cloned embryos

As mentioned above, somatic-like chromatin states can be transmitted to NT embryos and possibly result in inappropriate gene expression. It is especially clear that oocytes often fail to reverse silenced states of some embryonic genes in transplanted somatic chromatin because of layers of silencing mechanisms, including DNA methylation and H3K9 methylation. Apart from these types of reprogramming errors, there is evidence that active states of somatic genes are inherited in cloned embryos and the somatic type of gene expression continues in early embryos. In this section, we discuss examples of such abnormal somatic gene expression. For example, when myoblasts are used as donor cells for mouse NT, the myoblast cloned embryos start to express glucose transporter type 4 (Glut4), which is expressed in muscles but not in normal preimplantation embryos, at the late one-cell stage [154]. Similarly, the neuroectodermal marker gene is ectopically expressed in the endoderm cells in Xenopus NT embryos in which neuroectoderm nuclei are used as donors, and vice versa [160]. Interestingly, premature transcription of such somatic gene expression in NT embryos is also observed [160], suggesting that NT embryos are incapable of properly repressing some somatic genes of transplanted nuclei. Ng and Gurdon [62] provide a mechanistic insight into this epigenetic memory of somatic gene expression. Upon NT of somatic cells derived from somites (muscle precursors expressing a gene called MyoD), resulting embryos continue to aberrantly express MyoD. It has been shown that this memory of an active gene is dependent on trimethylation of lysine 4 on histone H3.3 in the promoter region of MyoD [62]. Another study proposes that a low level of histone H4K5 acetylation in somatic chromatin is inherited in cloned embryos and this may contribute to aberrant gene expression [127]. This idea needs to be further tested in individual genes that carry H4K5 acetylation. It is possible that more histone marks and chromatin proteins associated with active states of somatic gene expression would be discovered in future.

Other abnormalities in cloned embryos

Apart from the above mentioned chromatin defects, numerous other abnormalities have been reported in cloned embryos and animals, including defective placentas [161, 162], mitochondria heteroplasmy [17, 163], obesity [164], abnormal offspring syndrome [165], and short life spans [166]. In this section, we introduce other critical defects of cloned embryos, which are related to or derived from characteristics of somatic cells. In fertilised 1-cell mouse embryos, centromeres and pericentric heterochromatin regions of chromosomes associate with the periphery of the nucleolar precursor bodies [167, 168]. After NT of somatic nuclei into oocytes, the somatic type of heterochromatin often fails to be reprogrammed to the embryonic-like heterochromatin. Many cloned embryos show an abnormally high number of centromeres that do not associate with nucleolar precursor bodies [103]. Since centromeres are crucial for chromosome segregation, such abnormal positioning of centromeres may result in abnormal distribution of chromosomes to the daughter cells. In fact, cloned embryos often show improper distribution of chromosomes [169]. Recently, Mizutani et al. [65] demonstrated that abnormal chromosome segregation during early embryonic development is detrimental to full term development of cloned embryos. In relation to the abnormal chromosome segregation, somatic NT embryos exhibit abnormal spindle chromosome complex formation [170, 171] and spindle associating factors, such as calmodulin and NuMA, are found to be less abundant in somatic cell cloned embryos than in embryonic cell cloned embryos and fertilized embryos [170, 171]. Since defects in chromosome segregation seem to be one of the major causes of poor development of cloned animals, it would be interesting to determine to what extent abnormal heterochromatin and spindle formation affect early chromosome segregation defects.

Can we impose sperm-like states on somatic cells to increase the cloning efficiency?

As described above, there are a lot of differences between the sperm and somatic cells and each one of them, or a combination of these differences, may explain the low efficiency of cloning, as compared to efficient embryonic development after fertilisation. An ultimate goal in the reprogramming field is to increase the efficiency of animal cloning. It is reasonable to think that imposing sperm-like changes onto somatic cells will make them more prone to reprogramming. However, is such an approach feasible? In this section we discuss different possibilities of making somatic cells more like sperm.

Protamines

As mentioned above, the presence of protamines in the mature sperm is crucial for its ability to support correct embryonic development for at least a couple of reasons. Firstly, it may physically protect DNA from damage. Secondly, it may help to remove the majority of histones together with their marks and allow incorporation of oocyte-derived ones. Therefore, one could speculate that the easiest way to make a somatic cell more reprogrammable would be to wrap its DNA with protamines. However, it may be technically very challenging, because the deposition of protamines onto sperm chromatin is a complicated, multi-step process. It has been shown that impairments of single stages of this process (for example, removal of acetyl-lysine binding protein Brdt, or depletion of transition proteins) are detrimental for the sperm maturation and result in infertility [26, 45]. Therefore, in order to correctly deposit protamines on somatic cells, it is likely that one would not only need to introduce protamines into somatic cells, but also recapitulate other events occurring during spermiogenesis. Furthermore, this should be done in a sequential manner. Firstly, histone variants need to be expressed. The second step is to induce global histone acetylation, followed by histone removal and transition protein deposition. Introduction of protamines is only the last stage of the process. To summarise, if one could deposit protamines onto the chromatin of somatic cells, this might be a step forward in increasing the cloning efficiency. However, at the current state of methodology available, the correct deposition of protamines seems to be a technically challenging objective.

Carry-over proteins and RNAs

The mature sperm carries numerous proteins and RNAs which, as discussed above, could also be a part of sperm programming to support efficient embryonic development. Therefore, the next possible way to increase the efficiency of SCNT is to supply somatic cells with sperm proteins and RNAs. This would be challenging if one wants to isolate only the proteins and RNAs which are developmentally relevant, since numerous proteins and RNAs have been identified in the mature sperm and it is not well known which, if any, are important for embryonic development [72, 74]. A feasible approach is to isolate total protein and RNA from sperm and co-inject them into an oocyte together with a cell during the NT procedure. However, it cannot be excluded that the developmentally important proteins/RNAs are tightly associated with the sperm chromatin [74]. If this is the case, isolation of these factors could be technically demanding. Another approach is to directly deliver sperm into NT oocytes in which somatic

nuclei have been transplanted beforehand and subsequently remove the paternal pronucleus from the cloned embryos. This way, cloned oocytes are also activated by sperm injection in a similar way to normal fertilisation [97, 172]. However, this method did not improve cloning efficiency [172], which can be due to the fact that factors which are developmentally important may remain tightly associated with the sperm chromatin. Alternatively, enucleated early zygotes, in which sperm contents are delivered into recipient, can be used for NT [110, 111, 173].

Histone marks and DNA methylation

Another explanation for a sperm being superior to a somatic cell in its reprogramming capacity could be the unique pattern of its epigenetic modifications: histone and DNA methylation marks present on sperm chromatin can possibly pattern future embryonic gene expression. It is likely that imposing sperm-like epigenetic changes onto somatic cells would improve development of NT embryos. However, at the current state of knowledge this approach seems difficult. Firstly, so far the only genome-wide profiles in mouse and human sperm are available for H3K4me3, H3K27me3, H4K12ac, and DNA methylation [79, 80, 174]. It is known that epigenetic marks often work in combination and there are many more marks known than just these three; for example histone arginine methylation [175–177] or recently identified histone crotonylation [178]. It could be that the instructive information for embryonic development is a complex combination of various marks at various loci. If one can identify specific histone marks on a certain gene of sperm that are important for subsequent development, it would be worth trying to mimic such marks on somatic cell chromatin. Achieving gene locus-specific modification is currently difficult. However, with the development of zinc finger or TALE protein targeting approaches [179–184], it might be possible to induce sperm-like histone modifications in a gene-specific manner by targeting histone-modifying enzymes in the future.

Changing somatic cells into sperm?

The breakthrough study by Yamanaka and Takahashi [185] made it possible to derive pluripotent stem cells (iPS cells) from somatic cells by overexpression of transcription factors. iPS cells can differentiate into any type of cell in the body. Recently, Hayashi et al. [186] have succeeded in making spermatozoa from iPS and ES cells, and the produced spermatozoa supported full-term development after intracytoplasmic sperm injection. In theory it is therefore possible to obtain functional spermatozoa from somatic cells. However, this process requires multiple steps including dedifferentiation to an embryonic state, redifferentiation to primordial germ-like cells, and, ultimately, spermatogenesis. In addition, the resulting spermatozoa are haploid, carry paternal imprinting and require a maternal genome for successful development. Furthermore, the ultimate process of changing somatic cells into sperm—the spermatogenesis itself, is achieved by transferring primordial germ-like cells into testis. Therefore, this route may not be appropriate for the purpose of increasing cloning efficiency. However, this might be useful to produce a sperm deprived of a specific sperm factor important for subsequent development because gene knockdown or knockout can be achieved efficiently in pluripotent stem cells. An alternative route could be to directly change somatic cells to sperm-like or sperm progenitor-like cells by factor overexpression or extract treatment. This seems extremely challenging. Nevertheless, some progress has been made in inducing testis-specific gene expression in somatic cells treated with testis extracts. These extract-treated cells supported better development of cloned embryos than control non-treated cells [187]. It would be interesting to investigate why and how testis extract-treated cells are better reprogrammed in eggs/oocytes.

Conclusions and perspectives

Recent advances in genetic and epigenetic analytic approaches allowed the identification of some unique features of sperm chromatin that are absent in somatic cells. For example, histones are retained in sperm chromatin at promoter regions of developmentally important genes for subsequent gene expression in embryos. Such sperm chromatin modifications are likely to support embryonic development after fertilisation. Somatic chromatin does not have such "fine-tuning" for correct embryonic gene expression. Therefore, it is a likely explanation for why SCNT embryos often show abnormal reprogramming events compared to fertilised embryos. Somatic chromatin features, such as epigenetic memory, often remain in cloned embryos and can interfere with normal development. Moreover, other non-chromatin related factors, such as the presence of certain somatic-like transcription factors or presence of somatic-like spindle associating factors, can also impede the development of NT embryos. Therefore, it is remarkable that cloned embryos can sometimes develop to totally normal individuals. This is a good example illustrating that early embryonic development is characterised by both amazingly accurate programming and surprising plasticity. Nevertheless, there must be some essential requirements for the accomplishment of successful embryonic development. Identification of such roadblocks to development is a key challenge for

developmental and reproductive biology. Studying differences between the sperm and somatic cells in respect to their abilities to be reprogrammed by the eggs/oocytes would help to unravel the key requirements for successful development.

Conflict of interest   We have no conflict of interest.

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